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Differential innervation of superficial versus deep laminae of the dorsal horn by bulbo-spinal serotonergic pathways in the rat

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ABSTRACT

Convergent data showed that bulbo-spinal serotonergic projections exert complex modulatory influences on nociceptive signaling within the dorsal horn. These neurons are located in the B3 area which comprises the median raphe magnus (RMg) and the lateral paragigantocellular reticular (LPGi) nuclei. Because LPGi 5-HT neurons differ from RMg 5-HT neurons regarding both their respective electrophysiological properties and responses to noxious stimuli, we used anatomical approaches for further characterization of the respective spinal projections of LPGi *versus* RMg 5-HT neuron subgroups.

Adult Sprague-Dawley rats were stereotaxically injected into the RMg or the LPGi with the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHA-L). The precise location of injection sites and RMg vs LPGi spinal projections into the different dorsal horn laminae were visualized by PHA-L immunolabeling. Double immunofluorescent labeling of PHA-L and the serotonin transporter (5-HTT) allowed detection of serotonergic fibers among bulbo-spinal projections.

Anterograde tracing showed that RMg neurons project preferentially into the deep laminae V-VI whereas LPGi neuron projections are confined to the superficial laminae I-II of the ipsilateral dorsal horn. All along the spinal cord, double-labeled PHA-L/5-HTT immunoreactive fibers, which represent only 5–15% of all PHA-L-immunoreactive projections, exhibit the same differential locations depending on their origin in the RMg *versus* the LPGi.

The clear-cut distinction between dorsal horn laminae receiving bulbo-spinal serotonergic projections from the RMg *versus* the LPGi provides further anatomical support to the idea that the descending serotonergic pathways issued from these two bulbar nuclei might exert different modulatory influences on the spinal relay of pain signaling neuronal pathways.

1. Introduction

The rostral ventromedial medulla (RVM), which comprises the serotonergic region B3 with serotonin (5-hydroxytryptamine, 5-HT) - containing perikarya in the raphe magnus nucleus (RMg) and the lateral paragigantocellular reticular nucleus (LPGi), is known to play an important role in the bulbo-spinal descending control of nociceptive messages (Fields and Basbaum, 1978; Besson and Chaouch, 1987; Millan, 2002). Accordingly, both RMg and LPGi serotonergic neurons project directly into the dorsal horn of the spinal cord where is located the first central synaptic relay of nociceptive signals generated at peripheral nociceptors (Skagerberg and Björklund, 1985; Jones and Light, 1990, 1992). Furthermore, direct electrical stimulation of the RMg, that produces marked antinociceptive effects (Liebeskind et al., 1973; Oliveras et al., 1974), triggers a concomitant release of 5-HT at the spinal level (Bourgoin et al., 1980; Rivot et al., 1982; Hentall et al., 2006). The causal relationship between spinal 5-HT release and antinociception was clearly demonstrated in studies showing that the antinociceptive effect of RMg electrical stimulation could be suppressed in rats pretreated with the tryptophan hydroxylase inhibitor p-chlorophenylalanine, which produces a marked 5-HT depletion throughout the CNS (Rivot et al., 1980). On the other hand, the selective degeneration of bulbo-spinal serotonergic projections by the neurotoxin 5,7-dihydroxytryptamine was found to significantly alter the nocifensive behaviors associated with inflammatory (Yang et al., 2014) as well as neuropathic pain (Rahman et al., 2006), further confirming the implication of this descending pathway in pain modulatory mechanisms. More recently, novel shRNA interference approaches to selectively deplete 5-HT

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in these pathways provided clear-cut demonstration of 5-HT-mediated bulbo-spinal modulations of both acute and chronic pain in rats (Wei et al., 2010; Gautier et al., 2017), in convergence with a recent report on the effectiveness of selective optogenetic activation of RVM-serotonergic neurons to markedly affect pain signaling in rats (Cai et al., 2014).

In spite of such a large body of studies devoted to unveil the actual role of bulbo-spinal serotonergic pathways in the control of pain signaling, the situation is still confused to date. Thus, some authors (Suzuki et al., 2004; Rahman et al., 2006; Wei et al., 2010; Guo et al., 2014) concluded that these pathways are implicated in pain promoting mechanisms whereas others (Yamazaki et al., 1999; Hung et al., 2003; Gencer et al., 2015; Lee et al., 2015) reached the opposite conclusion, i.e. these pathways exerting an inhibitory influence on pain signaling.

One possible explanation of such discrepancies might be related to the existence of at least two different serotonergic pathways issued from the B3 region in rats. Indeed, as recalled above, this region comprises two separate clusters of serotonergic neurons, in the RMg and the LPGi, respectively. Interestingly, anatomical and electrophysiological studies provided evidence that 5-HT neurons in the RMg differ to some extent from those in the LPGi. Whereas serotonergic neurons in the RMg receive afferences from the ventral periaqueductal gray (PAG) (Fardin et al., 1984; Cameron et al., 1995; Hermann et al., 1997), those in the LPGi are innervated by projections coming mainly from the dorsal PAG (Fardin et al., 1984; Cameron et al., 1995; Normandin and Murphy, 2008). On the other hand, electrophysiological recordings by Gau et al. (2013) showed that LPGi serotonergic neurons are more intensely activated than RMg serotonergic neurons by thermal noxious stimulation (48–50°C). Accordingly, c-Fos immunolabeling showed a larger number of activated serotonergic neurons in the LPGi than in the RMg in response to thermal noxious stimuli (Gau et al., 2009).

In view of these differences at the level of cell bodies within the B3 region, our aim was to investigate whether differences also exist regarding the respective projections of RMg and LPGi serotonergic neurons into the dorsal horn of the spinal cord in rats. To this aim, we injected an anterograde tracer, the Phaseolus vulgaris leucoagglutinin (PHA-L), specifically into the RMg or the LPGi, and we used anti-PHA-L antibodies to visualize labeled fibers and terminals within dorsal horn laminae. Among these fibers, those with a serotonergic phenotype were characterized by double immunofluorescence labeling with anti-PHA-L and anti-serotonin transporter (5-HTT) antibodies. These investigations allowed the demonstration that specific dorsal horn laminae are reached by bulbo-spinal serotonergic fibers depending on their origin in the RMg or the LPGi, respectively.

2. Experimental procedures

2.1. Animals

Male Sprague-Dawley rats (body weight: 200–220 g, on arrival) were purchased from Janvier Breeding Center (53940 Le Genest Saint Isle, France). They were housed in a standard controlled environment (22 ± 1°C, 60% relative humidity, 12:12 h light-dark cycle, lights on at 7:00 a.m.) with food and water available *ad libitum*. Animals were allowed to habituate to these housing facilities without any handling for at least 1 week before being used for experiments. In all cases, experiments strictly followed the institutional guidelines that are in compliance with national and international laws and policies for use of animals in neuroscience research (Council Directive 86/609/EEC of the European Communities, November 24, 1986; Council Directive 87-848 of the French Ministère de l'Agriculture et de la Forêt, Service vétérinaire de la santé et de la protection animale, October 19, 1987). Data reported herein were obtained from studies specifically approved under registered nb 01296.02 by the Animal Research Committee of the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche.

2.2. Intra-bulbar injection of Phaseolus vulgaris leucoagglutinin (PHA-L)

Surgical procedures were performed in rats anesthetized with a combination of ketamine and xylazine (75 mg/kg and 12.5 mg/kg i.p., respectively) in saline (0.9% NaCl, total volume: 1.5 ml/kg i.p.) and mounted in a stereotaxic frame. The incisor bar was set at 18 mm below the horizontal plane passing through the interaural line, to incline the head by a 30° angle with respect to the horizontal plane at lambda and bregma level (Paxinos and Watson, 2005). With this head position, injections could be easily made along the rostro-caudal extension of B3 area. The scalp was incised at midline and a small opening was made into the atlanto-occipital membrane. PHA-L (Vector Laboratories, Burlingame, CA, USA; 10% in saline) was iontophoretically injected using a glass micropipette mounted with an angle of 45° (with respect to verticality) on the stereotaxic frame. The tip of the micropipette was moved down into the RMg (in 5 rats) or the LPGi (in 6 rats), at a point 200 μm posterior - 800 μm anterior or 400 μm posterior - 400 μm anterior to the obex, and 0 or 1200 μm lateral (on the right side) to the mid-sagittal plane, respectively. PHA-L solution was outflowed by direct current (3–5 μA) passing through the micropipette with a 15 s on/off cycle for 15 min. Once injection was achieved, the scalp was sewed up, and animals returned to their home cages after recovery from anesthesia.

2.3. Immunohistochemistry

2.3.1. Tissue preparation

Following a postoperative survival time of 20 days, rats were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with a heparin (25 IU/ml)-saline solution (during 3 min), followed by 0.12 M phosphate buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.05% picric acid for 25 min, and finally by 20% sucrose solution (during 10 min). The brain and spinal cord were removed and cryoprotected in 20% sucrose solution overnight. The next day, coronal sections of 50 μm thickness were cut using a freezing microtome and collected in containers filled with PBS. Four series of brain stem and spinal cord sections (at cervical C5, thoracic T5, lumbar L4-5, and sacral S3 levels) were made for each rat. They were processed in parallel with the same reagents, under the very same conditions for immunohistochemical labelings.

2.3.2. Immunohistochemical procedures

For PHA-L immunolabeling, sections were preincubated for 1 h in PBS containing 0.4% Triton X-100 and 1% normal goat serum (NGS), then incubated overnight with the primary antibody (rabbit anti-PHA-L, 1/2,000, AB_2313686, Vector Laboratories, Burlingame, CA, USA). After rinsing for 30 min with PBS, sections were incubated for 1 h with the secondary antibody (goat biotinylated anti-rabbit, 1/200, AB_2313606, Vector) in PBS containing 0.4% Triton X-100 and 1% NGS, then rinsed with PBS and incubated for 1 h in the avidin-biotin-horseradish-peroxidase solution (ABC Vectastain Elite Kit, Vector Laboratories). Sections were then processed in two series. The first series was stained (brown/orange) with 0.4% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO, USA) in PBS supplemented with H_2O_2 at progressively increasing concentrations (0.00025%, 0.0005%, 0.001%, 0.002%, 0.004%). The second series was first rinsed with 0.1 M Tris-HCl (pH 7.4, 15 min) and then